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Temperature- and pH-responsive aminopropyl-silica ion-exchange columns grafted with copolymers of *N*-isopropylacrylamide

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Abstract

We have designed copolymers of *N*-isopropylacrylamide, environmentally-responsive polymers, which respond to temperature and other external stimuli. In this study, we designed and synthesized copolymers that introduced ion-exchange groups. These copolymers responded to the temperature and the pH, and the copolymer-grafted aminopropyl silica beads were used as HPLC packing materials. This stationary phase altered the properties from hydrophilic to hydrophobic and from charge to non-charge by temperature and pH changes. We studied the separations of organic acids and phenylthiohydantoin-amino acids using environmentally-responsive chromatography, and confirmed the effects of the ion-exchange groups. The elution behaviors of these samples were controlled by the temperature changes without organic solvents in the mobile phase. It was confirmed that the interactions between the solute and stationary phase could be freely controlled by the temperature and the pH. Environmentally-responsive chromatography is expected to be applicable to the separation of pharmaceuticals and biomolecules, such as peptides, proteins and nucleic acids.
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Keywords: Stationary phases, LC; Temperature effects; pH effects; Poly(isopropylacrylamide); Amino acids, PTH derivatives

1. Introduction

Recently, various polymers have become known based on the development of the polymer science. Some types of polymers can sensitively detect environmental changes such as the pH, electric field, and temperature, which sharply and reversibly alter the structure and physical properties. Such stimuli-responsive materials are called intelligent polymers. Many new materials have been studied and applied to various fields, such as drug-delivery systems [1,2], cell-culture substrates [3,4] and actuators [5]. Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of the best-studied intelligent polymers, and is known to exhibit a thermally reversible phase transition at 32 °C. This transition temperature is called as the lower critical solution temperature (LCST). Below the LCST, the polymer chain of PNIPAAm is hydrated to an expanded form, which is soluble in water. PNIPAAm undergoes a reversible phase transition to an insoluble form, and dehydrates to a compact form above the LCST [6]. A functional surface which was grafted PNIPAAm chains reversibly changes from hydrophilic to hydrophobic properties based on temperatures [7]. We have developed a novel high-performance liquid chromatography (HPLC) system in which the hydrophilic–hydrophobic property changes when the stationary phase surface responds to external temperature changes [8,9]. We previously reported to have achieved the separations of peptides, proteins and bisphenol A using temperature-responsive chromatography [10,11]. We have designed a PNIPAAm copolymer, an environmentally-responsive polymer, which responds to the temperature and other external stimuli, and have demonstrated environmentally-responsive chromatography using HPLC packing materials modified with environmentally-responsive polymers [12].

In this study, we designed and synthesized copolymers of NIPAAm introduced ion-exchange groups. These

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copolymers respond to both the temperature and the pH. Copolymer-grafted aminopropyl silica beads were used as HPLC packing materials. The stationary phase surfaces altered the properties from hydrophilic to hydrophobic and from charge to non-charge based on the temperature and pH changes. Because many biological activities and pharmaceuticals have electric charge, it seems that such a system would be very useful for those separations. In the present work, we studied the separations of organic acids and phenylthiohydantoin (PTH)-amino acids using environmentally-responsive chromatography, and confirmed the effects of the ion-exchange group.

2. Experimental

2.1. Materials

N-Isopropylacrylamide (NIPAAm) was kindly provided by Kohjiin (Tokyo, Japan) and was purified by recrystallization from hexane. Butyl methacrylate (BMA) and *N*-*tert*-butylacrylamide (tBAAm) were purchased from Wako Pure Chemicals (Osaka, Japan) and Polysciences (Warrington, PA, USA). *N*,*N*-Dimethylaminopropylacrylamide (DMAPAAm; Kohjin) as a cationic ion-exchange group and acrylic acid (AAc; Wako) as an anionic ion-exchange group were purified by vacuum distillation, respectively. Aminopropyl silica beads (average diameter: 5 μm; pore size: 120 Å) were purchased from Nishio Industry (Tokyo, Japan). 2,2′-Azobisisobutyronitrile (AIBN; Wako) was purified by being recrystallized from ethanol. 3-Mercaptopropionic acid (MPA) and *N*,*N*-dimethylformamide (DMF) were purchased from Kanto Chemicals (Tokyo, Japan), and purified by vacuum distillation. Organic acids and PTH-amino acids were purchased from Wako. All other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of thermosensitive packing materials

PNIPAAm and its copolymers, which have reactive terminal functional groups, were synthesized with radical telomerization using a previously reported method [7,13]. The chain-transfer polymerization technique was employed using AIBN as an initiator and MPA as a chain-transfer agent, respectively. The syntheses of poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) [P(NIPAAm-*co*-BMA)] (IB) and poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate-*co*-dimethylaminopropylacrylamide) [P(NIPAAm-*co*-BMA-*co*-DMAPAAm)] (IBD) were as follows: NIPAAm (0.17 mol) and BMA (8.7 mmol) for IB copolymer preparations, and NIPAAm (0.15 mol), BMA (8.2 mmol) and DMAPAAm (8.6 mmol) for IBD copolymer each were dissolved in DMF with AIBN and MPA. After several freeze–thaw cycles to degas this solution, polymerization proceeded at 70 °C for 5 h. Polymers were recovered by precipitation in diethyl ether. Terminal carboxyl groups were esterified by *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide (molar ratio, 1:2.5:2.5) in ethyl acetate prior to a modification of the silica bead surfaces [14]. The active esterified polymer was dissolved in dry 1,4-dioxane and reacted with aminopropyl silica for 1 day. The reaction was repeated three times per batch using fresh reagents. PNIPAAm and its copolymer modified silica beads were thus obtained after extensive washing with methanol, distilled water, and Milli-Q water, successively. They were used as packing materials.

2.3. Transmittance measurements

LCSTs of PNIPAAm and its copolymers were determined by measuring the optical transmittance of a copolymer aqueous solution [8,9]. The transmittance of the polymer solutions was measured at 500 nm for various temperatures using a spectrometer (HITACHI U-3000). The temperature of the observation cells was controlled with a Lauda RC20 waterbath, with a deviation of ±0.02 °C. The LCST values for each copolymer were defined as the temperature where 50% optical transmittance of copolymer solutions was observed.

2.4. Preparation of P (NIPAAm-co-tBAAm-co-AAc) hydrogel-modified silica

Aminopropyl silica beads were grafted by a PNIPAAm hydrogel layer using a previously reported method [15,16]. 4,4′-Azobis(4-cyanovaleric acid) (ACV; Wako) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ; Tokyo Kasei Kogyo, Tokyo, Japan), an initiator and a condensing agent, respectively, were dissolved in DMF. Aminopropyl silica beads were immersed in the solution, and the reaction was carried out under an N_2 gas atmosphere. Modified silica beads were washed with DMF and ethanol, consecutively, and dried in vacuo overnight. Polymerization of PNIPAAm hydrogel on silica beads was carried out by the following method: NIPAAm (0.044 mol), tBAAm (4.9 mmol), AAc (1.5 mmol) and MBAAm (4.5 mmol) were dissolved in ethanol in a round-bottomed flask. Initiator-immobilized silica beads were added to the solution of monomers. The reaction mixture was then bubbled with N_2 gas, and polymerization was carried out at 70 °C for 15 h under an N_2 gas atmosphere. PNIPAAm hydrogel-modified beads were filtered and washed three times with methanol by decantation so as to remove any un-immobilized hydrogels. They were then dried in vacuo overnight, and the polymer hydrogel-modified beads were obtained.

Environmentally-responsive chromatography. The polymer-grafted silica beads were packed into a stainless-steel column (conventional column, 150 mm × 4.6 mm i.d.; short column, 50 mm × 4.6 mm i.d.). The column was connected to an HPLC system (Hitachi, Model L-6200 intelligent pump, L-4000 UV-monitor, D-2500 data processor). The column oven was a Shodex AO-30 (Showa Denko, Tokyo, Japan).

We used an IB and IBD copolymer-modified column to separate organic acids. An IBD copolymer-modified column and a hydrogel-modified column composed of NIPAAm, tBAAm and AAc (ItBA gel column) were used for the PTH-amino acids. The eluent was 1 mM (or 5 mM) NaCl aqueous solutions for the organic acids and phosphate buffered saline (PBS; pH 6.0; ionic strength, $I = 0.1$) for the PTH-amino acids. The elution behaviors of the samples were recorded at a flow rate of 1.0 ml/min at various temperatures and pH values.

Standard solutions of organic acids were prepared with formic acid, acetic acid, and propionic acid, and diluted to 0.01% by the eluent. PTH-amino acids were prepared with PTH-L-arginine hydrochloride standard (Arg; 1.00 mg/ml), PTH-L-asparagine standard (Asn; 0.40 mg/ml), PTH-L-aspartic acid standard (Asp; 1.08 mg/ml), PTH-glycine standard (Gly; 0.10 mg/ml) PTH-L-histidine hydrochloride standard (His; 1.00 mg/ml), PTH-DL-methionine standard (Met; 0.06 mg/ml), PTH-L-phenylalanine standard (Phe; 0.40 mg/ml), and PTH-L-valine standard (Val; 0.02 mg/ml).

3. Results and discussion

Fig. 1 shows the structures of the semitelechelic IB and IBD copolymers, and Fig. 2 illustrates a modified silica surface with PNIPAAm copolymer hydrogel. The polymer chains of PNIPAAm and its copolymers show an expanded conformation in water below the LCST due to strong hydration. Above the LCST, the polymer chains change to a compact form by sudden dehydration, because the hydrogen bond becomes unstable with elevated temperature [5]. To introduce hydrophobic sites into the PNIPAAm chains, the PNIPAAm LCST decreases with increasing polymer hydrophobicity [8,17], which enhances the dehydration of the polymer chains in aqueous media. By introducing hydrophilic sites, such as ion-exchange groups, the LCST shifts to a higher temperature in order to increase the hydrophilicity. The change in the temperature-dependent optical transmittance in 0.5% (w/w) aqueous solutions of PNIPAAm and its copolymers are shown in Fig. 3. PNIPAAm exhibits its LCST at 32.1 °C, while the LCST of an IB copolymer containing 5 mol% BMA (IB5%) shifts from 32.1 to 21.5 °C in order to introduce BMA as a hydrophobic group. In an IBD5% copolymer containing 5 mol% DMAPAAm and BMA (monomer ratio NIPAAm–BMA, 95:5), the LCST shifts higher than the LCST of IB5%. In addition, the LCST of an ion-exchange group introduced copolymers shifts due to pH changes [18,19]. It seems to alter the polarity of the polymer chain due to a change in the dissociation state of the ion-exchange groups. It is possible to choose the LCST by designing the polymer and modifying the pH.

Fig. 4 shows temperature-dependent retention profiles for organic acids on an IBD10% column and an IB5% column under a pH condition in which DMAPAAm and organic acids were dissociated. On the IBD10% column, the retentions of the organic acids increased below the LCST, and decreased the retention time above the copolymer's LCST. Therefore, there were no changes in the retention on the IB5% column, and the retention times of organic acids decreased on the IBD5% column (data not shown). This seems to be a result of the strong interaction working on the IBD10% column. Because it is indicated to work the interaction between organic acids and DMAPAAm, the interaction is reduced above the LCST. The charges of DMAPAAm as

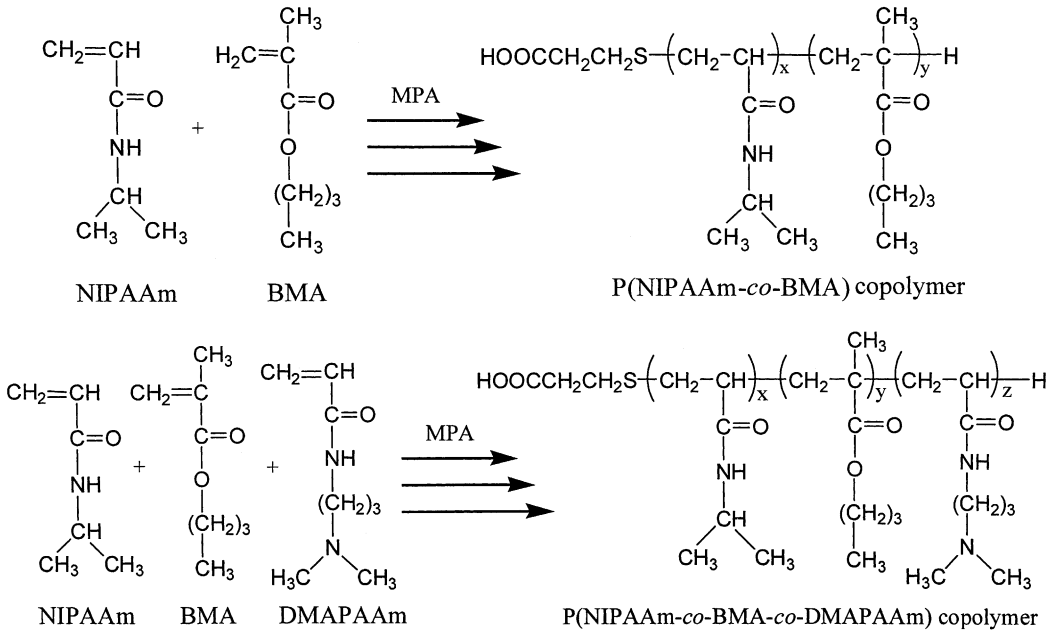

Fig. 1. Structures of P(NIPAAm-*co*-BMA) copolymer and P(NIPAAm-*co*-BMA-*co*-DMAPAAm) copolymer.

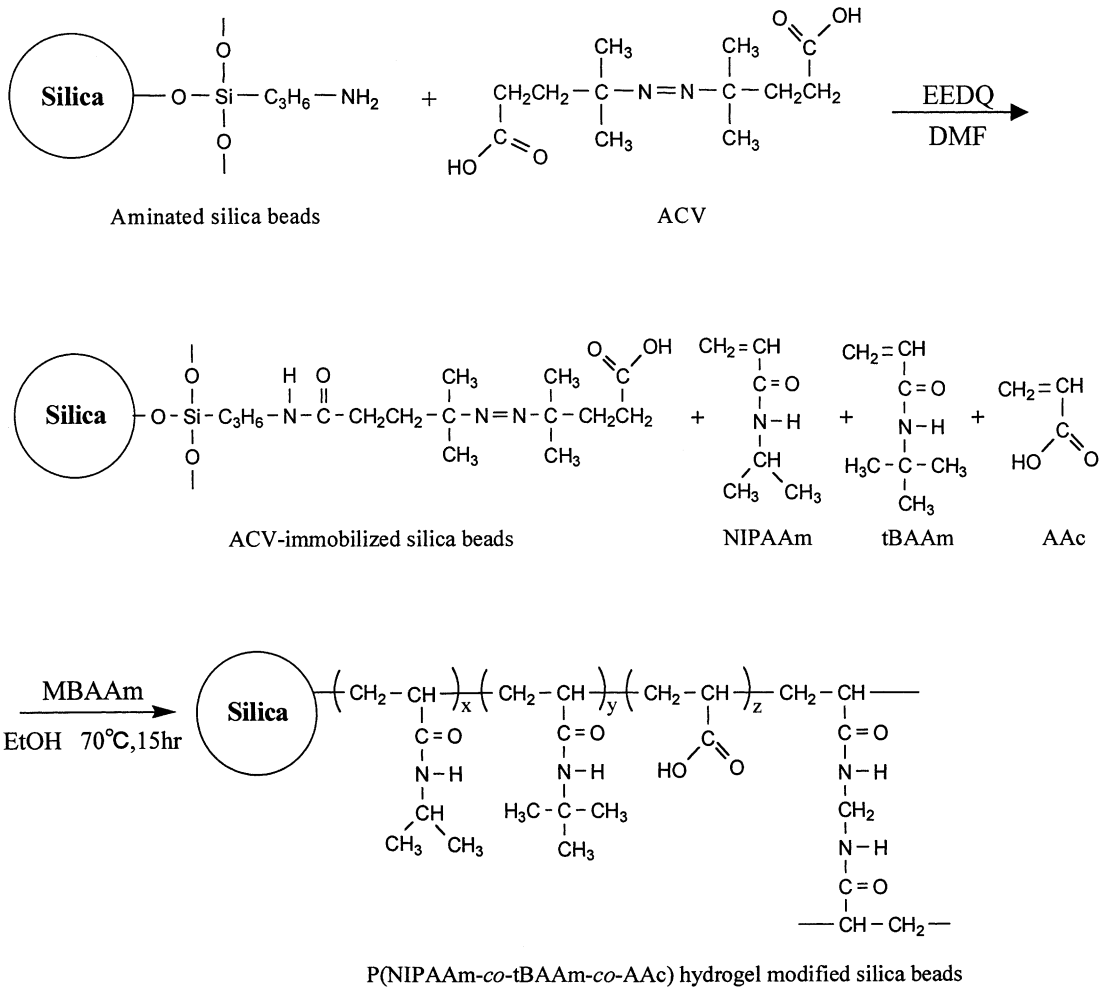

Fig. 2. Preparation scheme for modifying a silica surface with P(NIPAAm-*co*-tBAAm-*co*-AAc) hydrogel.

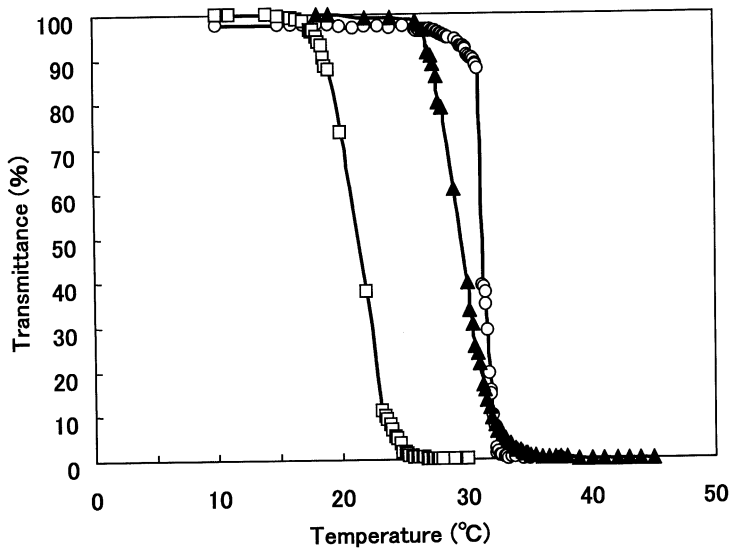

Fig. 3. Temperature-dependent optical transmittance changes of PNIPAAm (○), P(NIPAAm-*co*-BMA5%) (□), and P(NIPAAm- *co*-BMA5%-*co*-DMAPAAm5%) (▲).

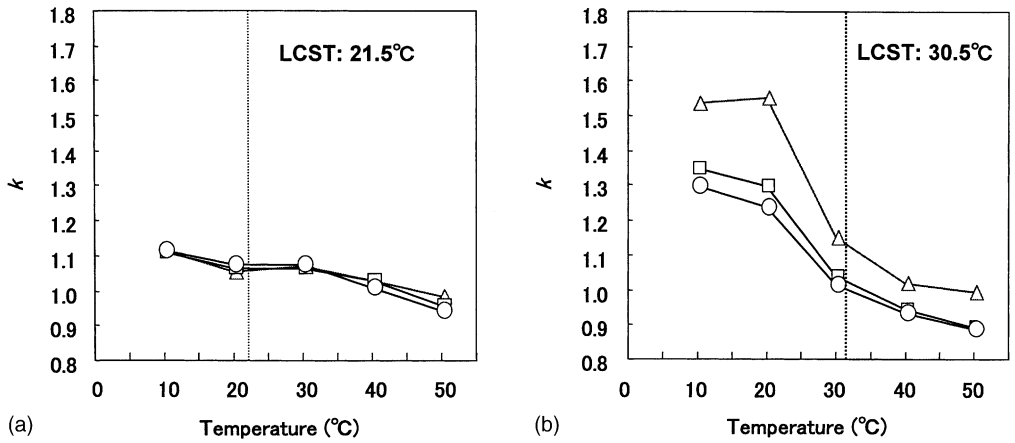

Fig. 4. Separation of organic acids on (a) P(NIPAAm-*co*-BMA5%) column, (b) P(NIPAAm-*co*-BMA5%-*co*-DMAPAAm10%) column. Samples: formic acid (△), acetic acid (□), propionic acid (○). Flow-rate: 1.0 ml/min; Eluent: 1 mM NaCl (pH 5.8); Detection: UV 215 nm.

an anion-exchange group appear on the polymer chain surface below the LCST, since the polymer chain is extended. However, above the LCST, the charges were hidden inside the polymer chain by compacting it. It seems that the electrostatic interactions between the organic acids and the stationary phase worked below the LCST. By raising the column temperature, the electrostatic interactions were reduced by hiding the charge; a decrease in the retention times of the organic acids was observed. As shown in Fig. 5, the stationary phase altered the properties from hydrophilic to hydrophobic and from charge to non-charge by temperature and pH changes in this environmentally-responsive chromatographic system. Table 1 gives the retention factors of organic acids on an IBD10% column at pH 3.0 and 6.0. At pH 3.0, lower than pK_a values of these organic acids, it was observed to decrease the retentions of the solute. The electrostatic interaction was reduced under this condition, because organic acids were undissociated. This suggested that the dissociative states of the solute or the stationary phase greatly affect the retentions. By using the pH and temperature changes, the elution behavior of the solute can be controlled in the present environmentally-responsive chromatographic system.

Table 1
The retention factors of organic acids on P(NIPAAm-*co*-BMA5%-*co*-DMAPAAm 10%) modified column at pH 3.0 and pH 6.0

	pH 3.0[a]		pH 6.0[a]	
	10 °C	50 °C	10 °C	50 °C
Formic acid	0.32	0.30	0.79	0.62
Acetic acid	0.06	0.06	0.87	0.62
Propionic acid	0.08	0.06	0.95	0.65

[a] 5 mM NaCl was used as mobile phase.

In the separation of PTH-amino acid using an ItBA column and an IBD column, the elution behaviors were varied based on the property of the amino acid. Table 2 gives the retention factors of PTH-amino acid at 10 and 50 °C. In a non-polar amino acid, such as Met, Phe and Val, the retention times were retarded with increasing the temperature on the IBD column and the ItBA column. However, the retention times of the basic amino acids,

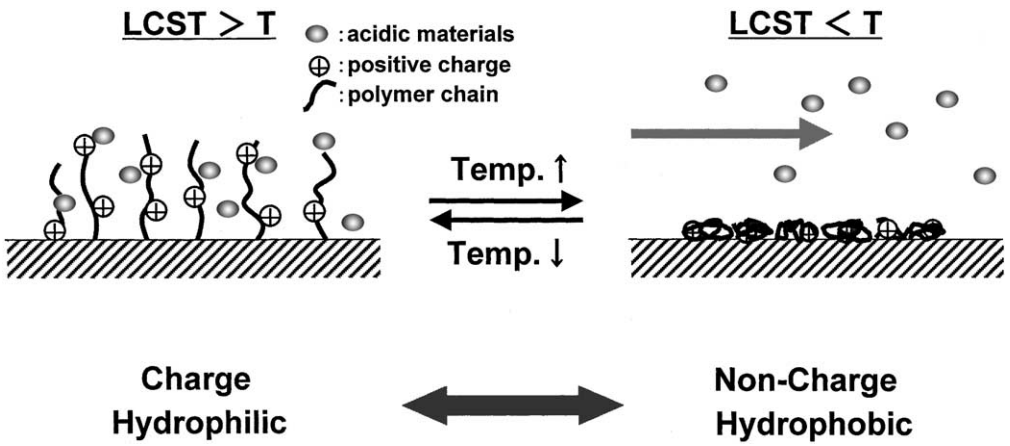

Fig. 5. Schematic illustration of environmentally-responsive chromatography.

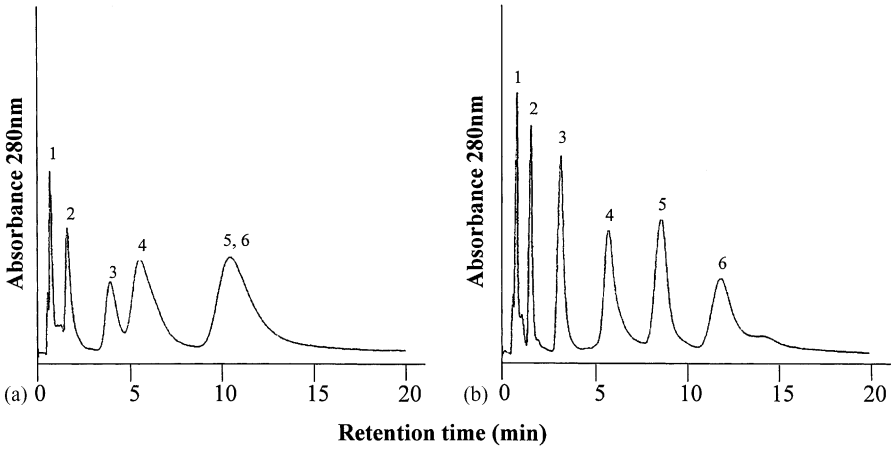

Fig. 6. Chromatograms of a mixture of PTH-amino acids at (a) 10 °C, (b) 50 °C on a P(NPAAm-*co*-tBAAm-*co*-AAc) gel column. Peaks: 1; Asp, 2; Asn, 3; His, 4; Met, 5; Arg, 6; Phe. Flow-rate: 1.0 ml/min; Eluent: PBS (pH 6.0, $I = 0.1$); Detection: UV 280 nm.

Table 2
The retention factors of PTH-amino acids using environmentally-responsive chromatography

Column	P(NIPAAm-*co*-tBAAm-*co*-AAc)		P(NIPAAm-*co*-BMA-*co*-DMAPAAm)	
	10 °C	50 °C	10 °C	50 °C
Val	5.20	6.98	0.26	0.59
Met	7.54	7.85	0.35	0.55
Phe	15.51	17.22	0.40	1.16
His	4.98	3.68	0.27	0.26
Arg	13.97	11.88	0.32	0.28

such as Arg and His, were reduced on the ItBA column. Thus, the retention times of the basic amino acids were slightly changed on the IBD column. This suggested that the elution behavior of the amino acids change because of the difference in the ion-exchange groups. We have previously achieved a simultaneous analysis of PTH-amino acids using temperature-responsive chromatography with water as a mobile phase [20]. In the present study, the results indicated that multiple interactions occurred simultaneously in the separation of the PTH-amino acids with environmentally-responsive chromatography. In non-polar amino acids, the retention times were retarded upon increasing the temperature. This suggested that the elution behaviors of non-polar amino acids occurred based on a hydrophobic interaction. On the other hand, the retention times of the polar amino acids were reduced on the ItBA column. It is suggested that electrostatic interactions between the polar amino acids and AAc occurred. Therefore, the peaks of Arg and Phe, which overlapped below the LCST, became to separate at an elevated temperature on the ItBA column, as shown in Fig. 6. It was possible to separate the basic and non-polar amino acids, such as Arg and Phe, by only increasing the temperature in the environmentally-responsive chromatographic system.

4. Conclusion

We developed a new method of chromatography, environmentally-responsive chromatography, and achieved the separation of organic acids and PTH-amino acids with an isocratic aqueous mobile phase. The elution behaviors of these samples were controlled by temperature changes without organic solvents in the mobile phase. It was confirmed that the interactions between the solute and the stationary phase could be freely controlled by the temperature and the pH. In addition, it was possible to control different interaction (hydrophobic interaction and electrostatic interaction) by changing only the temperature change in this system. Environmentally-responsive chromatography is expected to be applicable to the separation of pharmaceuticals and biomolecules, such as peptides, proteins and nucleic acids. Furthermore, it is possible to expand the analysis object by designing the polymers.

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